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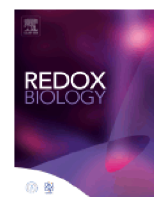

Research Paper

Lipid mediators involved in the oxidative stress and antioxidant defence of human lung cancer cells

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A B S T R A C T

Background: The oxidative modifications of bioactive macromolecules have important roles in carcinogenesis. Of particular interest are lipid peroxidation products, which are involved in the activation of Nrf2 and endocannabinoids that affect cancer progression.
Methods: In lung cancer tissues (squamous cell lung carcinoma - SCC and adenocarcinoma - AC), the glutathione peroxidase and catalase activity and glutathione level, together with the expression of Nrf2 and its activators/inhibitors were estimated. The oxidative modifications of DNA (8-hydroxy-2′-deoxyguanosine and *N7*-methylguanine), endocannabinoids (anandamide and 2- arachidonylglyceriol), their receptors (CB1/2, TRV1, GPR55), phospholipid fatty acids (arachidonic, linoleic and docosahexaenoic), and reactive aldehydes (4-hydroxynonenal, 4-oxononenal and malondialdehyde) were determined.
Results: Tumour tissues showed lower antioxidant capacity than healthy tissues, which was accompanied by lower levels of fatty acids and higher levels of reactive aldehydes. Disturbances in antioxidant capacity and enhanced DNA oxidative modifications were observed in 88% of AC patients and 81% of SCC patients. The 4-hydroxynonenal-Histidine adducts were detected in the necrotic and stromal cells in all tumours. These findings were associated with the enhanced Nrf2 activity, especially in AC. The strong difference between the cancer subtypes was evident in the levels of endocannabinoids, with an increase in 89% of SCC and a decrease in 85% of AC patients being observed. Additionally, the increase in the expression of CB1/2 receptors was observed only in 82% of AC, while the expression of VR1 and GPR55 was enhanced in 79% of SCC and 82% of AC patients.
Conclusions: This study shows significant differences in the redox status, Nrf2 pathway and endocannabinoid system between SCC and AC tissues. Understanding the relation between the various lipid mediators and antioxidants in different lung cancer subtypes may be beginning for further research on the effective anticancer therapy.

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Abbreviations: 2-AG, 2-arachidonylglyceriol; 4-HNE, 4-hydroxynonenal; 4-ONE, 4-oxononenal; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; AA, arachidonic acid; AC, adenocarcinoma; AEA, anandamide; AKT, serine/threonine-specific protein kinase; ALE, advanced lipoxidation-end products; ARE, antioxidant response element; Bach1, BTB and CNC homology 1, basic leucine zipper transcription factor; CAT, catalase; CB1/2, cannabinoid receptor type ½; COX-2, cyclooxygenase 2; Cul3, cullin-3; DGR, double glycine repeat domain; DHA, docosahexaenoic acid; EGFR, epidermal growth factor receptor; ERK1/2, mitogen-activated protein kinase 3/1; ESI, electrospray ionization; FAAH, fatty acid amide hydrolase; FAME, fatty acid methyl ester; GPR55, G protein-coupled receptor 55; GSH, glutathione; GSH-Px, glutathione peroxidase; GSSG, glutathione disulfide; HO-1, heme oxygenase 1; JNK1/2, c-Jun N-terminal kinases ½; KAP1, KRAB-associated protein-1; Keap1, kelch-like associated protein 1; LA, linoleic acid; LC-MS, liquid chromatography–mass spectrometry; LOD, limit of detection; LOQ, limit of quantification; MAGL, monoacylglycerol lipase; MDA, malondialdehyde; *N7*-MeG, *N7*-methylguanine; NFκBp52, nuclear factor kappa-light-chain-enhancer of activated B cells, subunit p52; Nrf 1/2/3, nuclear factor erythroid-2 related factor 1/2/3; NSCLC, non-small cell lung cancer; p21, protein 21; p62, protein 62; PBS, phosphate-buffered saline; PUFA, polyunsatutated fatty acids; ROS, reactive oxygen species; SCC, squamous cell lung carcinoma; TBST, tris-buffered saline and tween 20; TIMP-1, tissue inhibitor of matrix metalloproteinases-1; TRV1, transient receptor potential cation channel subfamily V member 1; UPLC-MS/MS, ultra-performance liquid chromatography, tandem mass-spectrometry

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1. Introduction

Lung malignancies are the leading cause of death in both men and women. More than 85% of lung cancers are non-small cell lung cancers (NSCLC), including the two most common histological subtypes of NSCLC: squamous cell lung carcinoma (SCC – approximately 30% of all lung cancers), and adenocarcinoma (AC – approximately 40% of all lung cancers, occurring more often in women than in men and in younger people than other subtypes of lung cancers) [1].

Cancer development is characterised by redox imbalance with a shift towards oxidative conditions. As a consequence, the oxidative modifications of cellular components, including phospholipids, DNA and proteins, are accumulated in tumour cells causing additional disturbances in their metabolism [2]. Several studies have shown that the lipid peroxidation products such as reactive aldehydes are involved in the intracellular signalling pathways and activation of transcription factors in cancer cells [3].

Nrf2 (nuclear factor erythroid-2 related factor 2) is one of the major transcription factors that are constitutively activated in many cancer cells [4]. Nrf2 is a cap 'n' collar basic leucine zipper transcription factor, which regulates the expression of antioxidant enzymes and several anti-apoptotic proteins, which confer cytoprotection against oxidative stress and apoptosis [5]. Nrf2 activates the transcription of target genes through binding to the antioxidant response element (ARE) found in those gene promoters. The genes regulated by Nrf2 encode antioxidants, xenobiotic metabolism enzymes and several ATP-dependent drug efflux pumps [6]. Therefore, the constitutive activation of Nrf2 in cancer promotes tumourigenicity and contributes to chemoresistance. However, the xenobiotic metabolism enzymes in conjunction with drug efflux proteins detoxify cancer drugs, whereas antioxidants provide cytoprotection by attenuating drug-induced oxidative stress and apoptosis [7]. Therefore, chemotherapy for advanced, inoperable NSCLC is generally palliative.

Nrf2 activity is regulated by its cytoplasmic anchor – Keap1 (Kelch-like ECHassociated protein 1) that forms a complex with the transcription factor. Several studies have shown that the constitutive activation of Nrf2 in lung cancer is strongly connected with mutations in the *Keap1* gene [8,9]. In physiological conditions, Keap1 constitutively suppresses Nrf2 activity; however, electrophilic compounds hamper the Keap1-mediated proteasomal degradation of Nrf2, leading to the transcriptional induction of target genes that ensure cell survival [10]. It was found that in the case of lung cancer, mutation of a glycine to cysteine in its DGR domain reduces the affinity of Keap1 for Nrf2 [11]. It was found that Keap1 is a cysteine-rich protein that is highly susceptible to electrophilic molecules, such as reactive oxygen species (ROS) or reactive aldehydes, which are the products of lipid peroxidation (e.g., 4-HNE). Their interactions with Keap1 lead to changes in its confirmation and thereby to Nrf2 activation. Complexes of 4-HNE-Keap1 were detected in NSCLC [12]. It was also observed that 4-HNE occurs at a higher level in human lung cancer cells and acts as a signalling molecule promoting tumour cell viability [13]. Other lipid mediators, such as endocannabinoids like anandamide (AEA) and 2-arachidonylglyceriol (2-AG), are also known to affect the progression of cancer development [14], but the exact mechanism of their action is unknown. Endocannabinoids belong to the endocannabinoid system, which is involved in the reaction of cancer cells to the generation of higher levels of reactive oxygen species [15]. Moreover, natural and synthetic cannabinoids as well as their receptors, CB1, CB2, TRV1, and GPR55, and enzymes involved in the endocannabinoid metabolism have been reported to affect cancer growth at more than one step in several subtypes of malignancies [16]. Accordingly, previous studies identified the anti-tumourigenic activities of cannabinoids, such as inhibition of tumour cell proliferation [17] and angiogenesis [18], as well as induction of apoptosis and autophagy [19]. Cannabinoids and endocannabinoid-related compounds were able to affect lung cancer cell proliferation, induce apoptosis, and inhibit migration and invasiveness [20]. In NSCLC, cannabinoids inhibit cancer cell invasion *via* increasing the expression of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) [21]. It was shown that the endocannabinoid system components protect cancer cells against the higher levels of reactive oxygen species [15] and affect Nrf2 activity [22].

Therefore, the aim of this study was to evaluate the relationship between the lipid mediators and activity of the transcription factor and compare these relationships in the two main subtypes of human NSCLC: squamous cell lung and adenocarcinoma. Finding and understanding the differences in the mechanisms of Nrf2 activation in these cancers may be applied in anticancer therapies.

2. Material and methods

Surgically resected non-small cell lung cancer [NSCLC] and adjacent normal tissue specimens were collected from a group of 28 female and 44 men with a mean age of 62 (46 – 77) years. All patients underwent pulmonary resection for primary NSCLC in the Department of Thoracic Surgery of the Medical University of Bialystok, Poland and the University of Zagreb School of Medicine, Clinical Hospital Centre Zagreb, Division of Pathology, Croatia. Thirty eight samples from patients with pulmonary squamous cell carcinoma (SCC) (12 female and 26 men with mean age of 62 ((46 – 77)years)) and 34 samples from patients with adenocarcinoma (AC) (16 female and 18 men with mean age of 60 ((50 – 72) years)) were collected.

The inclusion criteria for the current study were the following: original diagnosis of lung AC or SCC based on the histologic evidence of glandular differentiation or squamous differentiation, respectively; *completely resected tumour (free resection margins);* stage I or stage II NSCLC; a minimum of three-year follow-up including monitoring for events of cancer recurrence and lung cancer–related death; availability of representative fresh-frozen tumour specimens (the material containing at least 50% tumour cells for DNA extraction); and no adjuvant chemotherapy. This study was approved by the Institutional Ethical Committee of the Medical University of Bialystok as well as the University of Zagreb School of Medicine and informed consent was obtained from each patient. The exclusion criteria were as follows: lack of written consent; recent treatment with certain medications, including nonsteroidal anti-inflammatory drugs, steroids, and oral contraceptives; alcohol abuse; and heavy smokers.

A piece of each collected tissue was homogenised under standardized conditions; 10% homogenates were centrifuged at $10{,}000 \times g$ for 15 min at 4 °C, and the supernatants were stored at –80 °C and used for the estimation of biochemical parameters.

2.1. Antioxidant parameters

2.1.1. Determination of glutathione peroxidase activity

Glutathione peroxidase (GSH-Px – EC.1.11.1.6) activity was assessed spectrophotometrically using the method of Paglia and Valentine [23]. GSH-Px activity was assayed by measuring the conversion of NADPH to NADP. One unit of GSH-Px activity was defined as the amount of enzyme catalysing the oxidation of 1 μmol NADPH min^{-1} at 25 °C and pH 7.4. Enzyme specific activity was expressed in units per mg of protein.

2.1.2. Determination of catalase activity

Catalase (CAT – EC.1.11.1.9) activity was determined by

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